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Purification and Characterization of Phytase from Bran of *Triticum aestivum* L. cv. Nourin #61

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Two phytase isozymes (PHY1 and PHY2) were purified homogeneously from bran of *Triticum aestivum* L. cv. Nourin #61 by $(NH_4)_2SO_4$ fractionation, methanol fractionation, Sephacryl S-200 HR gel filtration chromatography, DEAE-TOYOPEARL chromatography, CM-TOYOPEARL chromatography and second Sephacryl S-200 HR gel filtration chromatography. Molecular weights of the two enzymes were 71,000 and 66,000 by gel filtration, and 68,000 and 66,000 by SDS-PAGE, respectively. Optimum pH and temperatures were 6.0 and 45°C for PHY1, and 5.5 and 50°C for PHY2. The activity of both phytases was stable at pHs between pH 4.0-7.0 and below 40°C. The K_m values for *myo*-inositol hexakisphosphate (IHP) were 0.48 μM for PHY1 and 0.77 μM for PHY2. The K_i values for Pi were 2.69 mM for PHY1 and 6.59 mM for PHY2. Both phytases showed relatively high specificity for IHP.

Keywords: phytase, phytic acid, wheat bran, Nourin #61, purification

Phytic acid (*myo*-inositol hexakisphosphate; IHP) is an abundant plant constituent, which is reported to comprise 1-5% of the weight of edible legumes, cereals, oil seed, pollens and nuts (Graf *et al.*, 1987). IHP is one of several strong metal-chelating agents, and its action has both positive and negative effects, depending on the system. It binds minerals and markedly decreases mineral bioavailability *in vivo*. If the diets of animals with simple stomachs such as pigs or poultry which do not have a hydrolysis system for IHP *in vivo* contain an abundant amount of IHP, those animals may suffer from mineral deficiency, resulting in inhibited physical development. Simons *et al.* (1990) described that the addition of microbial phytase to the diets of pigs and broilers improves the phosphorus availability in their diets. The iron-chelating agent, IHP, on the other hand, greatly accelerates the reduction of Fe^{2+}-mediated active oxygen, yet strongly blocks the generation of the iron-driven hydroxyl radical and suppresses the peroxydation of lipids by chelating iron ions (Graf *et al.*, 1987). High concentrations of IHP also prevent the browning and putrefying of various foods by inhibiting polyphenoloxidase (Graf *et al.*, 1987). Many scientists have evaluated the association of IHP with human diseases. It is hypothesized that IHP reduces the risk of cancer (Graf & Eaton, 1985): it inhibits cell-oxidation in the human cell which induces senescence, and hence reduces the formation of a tumor. Graf and Eaton (1985, 1990) mentioned that IHP in diets can lower the incidence of colonic cancer and protect against other bowel diseases, and it also has preventive effects against various other diseases (renal calucui, hypercalciuria, kidney stones, platelet aggregation, organ ischemia, homolytic anemia, pulmonary insufficiency, erythrocytosis, hyperlipidemia and diabetes).

Phytase (*myo*-inositol-hexakisphosphate phosphohydrolase) catalyzes the hydrolysis of IHP into inositol mono-, di-, tri-, tetra- and penta- phosphates (IP1-5) and inorganic phosphates (Pi). Elucidation of characterization and structure of phytase is necessary to understand the function of IHP and its degradation products. Phytase has been investigated in various plants and microorganisms, however, it has only been purified from cotyledons of soybean (Gibson & Ullah, 1988), maize (Laboure *et al.*, 1993), *Aspergillus ficuum* (Ullah & Gibson, 1987) and *Escherichia coli* (Greiner *et al.*, 1993). The complete amino acid sequence has also been determined in some microbial phytases (e.g. *A. ficuum* (Ullah & Dischinger, 1993; Van Hartingsveldt *et al.*, 1993)). Among plant phytases the only complete amino acid sequence of maize has been found in a GenBank protein sequence data base (Maugenest *et al.*, 1997).

Wheat bran contains an abundant amount of IHP and has a high activity of phytase (Cosgrove, 1980; Graf & Eaton, 1990). Nagai and Funahashi (1962, 1963) and Lim and Tate (1971, 1973) described the characterization of phytase from wheat bran, however, they did not purify the homogeneous enzyme, and did not specify the cultivated variety of wheat. In a previous study we purified two phytases homogeneously from wheat bran which were mixed with Dark Northern Spring (DNS) from the U. S. A. and Western Red Spring (WRS) from Canada (Nakano *et al.*, 1997), although we could not determine whether they were from DNS or WRS or both. In this study, we purified phytase from a bran of a single cultivated variety of wheat, Nourin #61 from Japan. Differences in the properties of the phytases of this variety and the mixture with DNS and WRS are discussed.

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Abbreviations: IHP, *myo*-inositol hexakisphosphate; DNS, Dark Northern Spring; WRS, Western Red Spring; Pi, inorganic phosphates.

Materials and Methods

Materials Wheat bran was obtained from Maruei Seifun Co., Ltd. (Niigata) and stored at 1-4°C. The cultivated variety was *Triticum aestivum* L. cv. Nourin #61 produced in Japan.

Enzyme assay Phytase activity was assayed in a 250 μl of reaction mixture containing 250 mM acetate buffer (pH 5.5), 15 mM Na-IHP (Sigma, St. Louis, Mo.), and enzyme solution. The reaction mixture was incubated at 37°C for 15 min, and the reaction was stopped by adding 250 μl of 10% (w/v) trichloroacetic acid, and the liberated Pi was measured by the method of Fiske and Subbarow (1925). One unit of enzyme activity was defined as the amount of enzyme which liberates 1 μmol of Pi from the substrate per minute under the assay conditions. The protein solution was estimated from the absorbance at 280 nm or by the method of Bradford (1976) using albumin bovine serum as the protein standard.

Purification of phytase All operations were carried out at 0-4°C.

About 1.2 kg of wheat bran was steeped in 4 *l* of cold distilled water. After over night storage, the mixture was squeezed through double gauze. The filtrate was centrifuged at 15,000×*g* for 15 min, and the supernatant was used as a crude enzyme solution.

Solid ammonium sulfate was added to the crude enzyme solution up to 40% saturation. The mixture was permitted to stand for 1 h and centrifuged at 15,000×*g* for 15 min. The supernatant was brought to 70% saturation of ammonium sulfate. The precipitate was collected by centrifugation at 15,000×*g* for 15 min, dissolved in a small volume of distilled water, and dialyzed against distilled water overnight; the insoluble matter was centrifuged off.

Cold methanol was added to the supernatant up to 45% (v/v) concentration. The formed precipitate was removed after centrifugation at 15,000×*g* for 15 min and the supernatant was brought up to 65% (v/v) concentration of methanol. The precipitate was collected after centrifugation and dissolved in a small volume of distilled water, then dialyzed against distilled water overnight. The insoluble matter was centrifuged off and concentrated using polyethyleneglycol as described by Pohl (1990).

Enzyme solution dialyzed against 20 mM Tris-maleate buffer (pH 6.0) was passed through a Sephacryl S-200 HR (Pharmacia, Uppsala) column (2.5×115 cm) which was equilibrated with 20 mM Tris-maleate buffer (pH 6.0) containing 1 M NaCl and 0.1% (v/v) Triton X-100. Flow rate was 1.5 ml/min, and the eluate was collected in 4 ml fractions. The active fractions were combined and concentrated using polyethyleneglycol, then dialyzed against 20 mM Tris-HCl buffer (pH 7.0) containing 0.1% (v/v) Triton X-100.

The enzyme solution was loaded on a DEAE-TOYOPEARL (Tosoh, Tokyo) anionic-exchanger column (2.5×13.5 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.0) containing 0.1% (v/v) Triton X-100 at a flow rate of 1.0 ml/min. The column was thoroughly washed with the same buffer and the adsorbed protein was eluted from the column with a linear gradient of 200 ml of the same buffer in the mixing chamber and 200 ml of the buffer containing 0.4 M NaCl in the reservoir. The eluate was collected in 4 ml fractions. Non-adsorbed fractions with phytase activity were combined, concentrated using polyethyleneglycol and dialyzed against 20 mM acetate buffer (pH 5.0) containing 0.1% (v/v) Triton X-100, then used for further purification.

The enzyme solution was loaded on a CM-TOYOPEARL (Tosoh) cationic-exchanger column (1.5×26.0 cm) equilibrated with 20 mM acetate buffer (pH 5.0) containing 0.1% (v/v) Triton X-100 at a flow rate of 0.5 ml/min. The column was thoroughly washed with the same buffer and the adsorbed protein was eluted from the column with a linear gradient of 200 ml of the same buffer in the mixing chamber and 200 ml of the buffer containing 0.4 M NaCl in the reservoir. Phytase activity was eluted with about 0.2 M NaCl. The eluate was collected in 3 ml fractions. The active fractions were combined, concentrated using polyethyleneglycol, and then dialyzed against distilled water.

The active fraction was passed through a Sephacryl S-200 HR column (2.5×115 cm) which was equilibrated with 20 m M Tris-maleate buffer (pH 6.0) containing 1 M NaCl and 0.1% (v/v) Triton X-100. Flow rate was 1.5 ml/min, and the eluate was collected in 1.5 ml fractions. The phytase activity was detected from fractions No. 40 to No. 72.

Electrophoresis Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemli (1970).

Estimation of molecular weight Molecular weight of the enzyme was estimated by SDS-PAGE and Sephacryl S-200 HR gel filtration chromatography.

N-terminal amino acid sequence After SDS-PAGE, the protein of interest was transferred to clear blot membrane-P (ATTO, Tokyo) by electroblotting. Amino acid sequence was determined with an amino acid sequencer (model 473A, Applied Biosystems, Norwalk, Ct.)

Results

Purification A summary of the purification is given in Table 1. As shown in Fig. 1, the phytase activity was separated by DEAE-TOYOPEARL chromatography into a major peak (non-adsorbed fractions) and a minor one (adsorbed fractions). Phytase in the non-adsorbed fractions was further purified by CM-TOYOPEARL chromatography and Sephacryl S-200 HR gel filtration. In the second Sephacryl S-200 HR gel filtration, two adjacent active peaks were detected (fractions No. 51 and 54) (Fig. 2). SDS-PAGE analysis of active fractions revealed that fractions No. 49-55 near the peaks contained two polypeptides but that fractions No. 42-48 and No. 56-68 contain only one of either polypeptide. Fractions No. 42-48 and No. 56-68 were combined separately and designated PHY1 and PHY2, respectively. PHY1 and PHY2 were purified about 400-fold and 440-fold achieved with recovery of about 1.4% and 1.9% from the crude enzyme solution, respectively. The homogeneity of PHY1 and PHY2 was confirmed again by SDS-PAGE (Fig. 3).

Molecular weight and N-terminal amino acid sequence The molecular weight of PHY1 and PHY2 was estimated to be approximately 68,000 and 66,000 by SDS-PAGE, and approximately 71,000 and 66,000 by Sephacryl S-200 HR gel filtration of the native enzyme, respectively. Consequently,

Table 1. Summary of purification of phytases from bran of *T. aestivum* L. cv Nourin #61

Step	Activity (Units)	Protein (mg)	Specific act. (Units/mg)	Purification (fold)	Yield of act. (%)
Crude enzyme	2866.8	4385.5	0.7	1.0	100.0
40–70% $(NH_4)_2SO_4$ fraction	2037.3	1632.8	1.2	1.9	71.1
45–65% Methanol fraction	1694.0	389.1	4.4	6.7	59.1
First Sephacryl S-200 HR	696.0	35.7	19.5	29.8	24.3
DEAE-TOYOPEARL	496.9	9.5	52.0	79.6	17.3
CM-TOYOPEARL	341.1	2.8	122.7	187.6	11.9
Second Sephacryl S-200 HR PHY1	39.6	0.2	260.5	398.6	1.4
PHY2	55.1	0.2	288.4	441.2	1.9

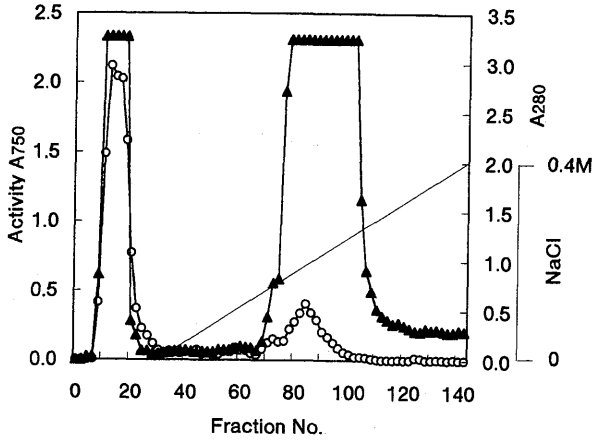

Fig. 1. DEAE-TOYOPEARL column chromatography of phytase from bran of *T. aestivum* L. cv Nourin #61. ○, phytase activity; ▲, A280; —, concentration of NaCl.

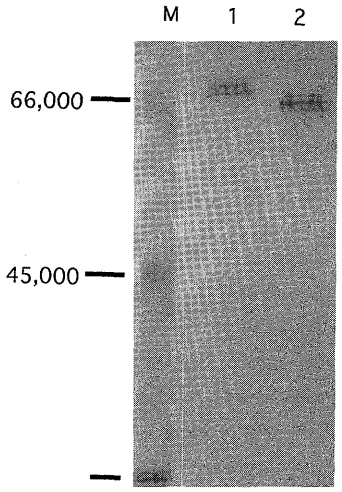

Fig. 3. SDS-polyacrylamide gel electrophoresis of phytase from bran of *T. aestivum* L. cv Nourin #61 after second Sephacryl S-200 HR gel filtration. Lanes: 1, PHY1; 2, PHY2; M, molecular marker. The gel was stained by silver staining method.

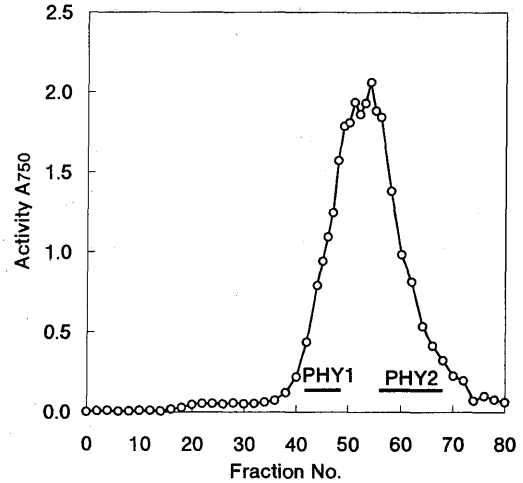

Fig. 2. Sephacryl S-200 HR gel filtration column chromatography of phytase from bran of *T. aestivum* L. cv Nourin #61. ○, phytase activity; —, the fractions recovered as PHY1 and PHY2.

Table 2. Summary of characterization of phytases from bran of *T. aestivum* L. cv Nourin #61.

	PHY1	PHY2
M.W. Sephacryl S-200 HR	71,000	66,000
SDS-PAGE	68,000	66,000
Optimum pH	6.0	5.5
Stable pH range	4–7	4–7
Optimum temperature (°C)	45	50
Stable temperture (°C)	40	40
K_m value for IHP (μM)	0.48	0.77
V_{max} (μmol/mg/min)	127.0	242.1
K_i value for Pi (mM)	2.69	6.59

both PHY1 and PHY2 are monomeric proteins.

The N-terminal amino acid sequences of PHY1 and PHY2 were detected as: EPAXTLTGPSRPV. No difference could be found between the sequences of the two phytase isozymes. These sequences were compared with protein sequences entered in the Swiss-Plot data base, but no homologous sequence was observed.

Effects of pH on the enzyme activity and stability

Enzyme activity at various pHs was measured by the method described in Materials and Methods except for buffers: 0.2 M acetate-HCl buffer between pH 3.0–4.0, 0.2 M acetate buffer between pH 4.0–5.5, and 0.2 M tris-maleate buffer between pH

Table 3. Substrate specificity of phytases from bran of *T. aestivum* L. cv Nourin #61.

Substrate	Relative activity (%)	
	PHY1	PHY2
IHP	100	100
β-Glycerophosphate	59	84
p-Nitrophenylphosphate	51	211
Glucose-6-phosphate	5	29
Adenosine 5′-monophosphate	31	24
ADP	46	35
ATP	92	169

5.5–8.0. The pH-activity curve of PHY1 was different from that of PHY2. Each optimum-pH was 6.0 for PHY1 and 5.5 for PHY2 (Table 2). Enzyme stability at various pHs was examined by measuring the activity after 120 μl of enzyme solution was diluted with 200 μl of each of the above buffers and incubated at 4°C for 12 h. Both of the enzymes were stable between pH 4.0–7.0 (Table 2).

Effects of temperature on the enzyme activity and stability For PHY1, the optimum temperature was 45°C. Between 35–50°C, 80% of the activity was retained, compared to 100% activity at 45°C (Table 2). On the other hand, the optimum temperature for PHY2 was 50°C and between 35–55°C 90% of the activity was retained, compared to 100% activity at 50°C (Table 2). For the thermal stability assay, 30 μl of enzyme solution was diluted with 75 μl of 0.5 M acetate buffer (pH 5.5), and incubated for 15 min at various temperatures. After the diluted solution was held at 4°C for 1 h, the activity was measured. Both phytases retained high activity up to 40°C (Table 2).

Substrate specificity The substrate specificities of PHY1 and PHY2 were investigated by the procedure of Nagai and Funahashi (1962). The relative rates of hydrolysis are summarized in Table 3. PHY1 and PHY2 hydrolyzed not only IHP but also several other phosphate esters, though there were differences in the relative activities. The hydrolysis rate for IHP of PHY1 was the highest among the substrates tested. ATP was also hydrolyzed at a high rate, but the hydrolysis rates for other phosphorylated compounds did not reach 60% of that for IHP. PHY2, however, hydrolyzed *p*-nitrophenylphosphate and ATP at a higher rate than IHP. The hydrolysis rate for β-glycerophosphate was also high, but those for others were less than 35% of the rate for IHP. The K_m values for IHP were calculated as 0.48 μM (PHY1) and 0.77 μM (PHY2), respectively, by Lineweaver-Burk plots.

Effect of metal ions on the phytase activity Phytase activity was assayed in 250 μl of a reaction mixture containing 250 mM acetate buffer (pH 5.5), 15 mM Na-IHP, 10 mM metal ions and 0.2 units of enzyme solution as described by Hayakawa *et al.* (1989). Both phytases were strongly inhibited by $Mo_7O_{24}^{6-}$, Hg^{2+}, Ag^{+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+} and F^{-} (Table 4). Ca^{2+} and Mg^{2+} inhibited the activity of PHY2 more than PHY1. K^{+} was not an effective agent against the activity of PHY2, but could inhibit 16% of the activity of PHY1. The K_i values for Pi were calculated as 2.69 mM (PHY1) and 6.59 mM (PHY2) respectively by Lineweaver-Burk plots.

Table 4. Effect of metal ions on the activity of phytase from bran of *T. aestivum* L. cv Nourin #61.

Substance (10 mM)	PHY1	PHY2
Control	100	100
$ZnSO_4$	12	14
$MnCl_2$	66	77
$HgCl_2$	0	0
$FeCl_2$	0	0
$CuSO_4$	10	16
$AgNO_3$	0	0
$FeCl_3$	19	27
$(NH_4)_6Mo_7O_{24}$	18	15
$CaCl_2$	87	48
$MgCl_2$	58	39
NaF	26	38
LiCl	71	69
KCl	84	105
KNO_3	29	53
ICH_2COOH	67	75

Discussion

In this study we purified two phytases (PHY1 and PHY2) homogeneously from the bran of *T. aestivum* L. cv. Nourin #61, a domestic variety. The purification was achieved basically by the same method as that used for phytases (PDW1 and PDW2, described in the previous study as Phy1and Phy2, respectively) from bran of the mixture of DNS and WRS (Nakano *et al.*, 1997). By DEAE-TOYOPEARL anionic exchanger column chromatography, the phytase activity was detected in adsorbed and non-adsorbed fractions, but the activity of the latter fractions was much higher than that of the former. We conclude that the enzyme in adsorbed fractions was not phytase but acid phosphatase, because the adsorbed fractions hydrolyzed *p*-nitrophenylphosphate and IHP at a higher and lower rate, respectively, than the non-adsorbed fractions. Therefore, only non-adsorbed fractions were used for the next purification step. Partial modifications of the procedure were needed, for example, a change of fractionation range in ammonium sulfate and methanol precipitations, and an addition of Triton X-100 to the buffer used in chromatography to stabilize of the enzyme. These suggest that the properties of phytases somewhat differ between varieties. A detailed comparison of their properties is described below.

Molecular weights of two purified phytases, PHY1 and PHY2, were determined by gel filtration and SDS-PAGE: PHY1, 71,000 by gel filtration and 68,000 by SDS-PAGE; PHY2, 66,000 and 66,000; this suggests that both PHY1 and PHY2 are monomeric proteins in a similar manner to PDW1 and PDW2. However, the molecular weights of PHY1 and PHY2 were smaller than PDW1 and PDW2 (73,000 and 72,000), and larger than that of the phytase (F1 and F2) from wheat bran reported by Lim and Tate (47, 000) (1973). Two phytases were detected not only in the mixture of DNS and WRS, but also in the single variety Nourin #61, and N-terminal amino acid sequences of PHY1 and PHY2 were identical with PDW1 and PDW2 as far as we could determine. Therefore, we hypothesize that two phytases always exist in a single variety of wheat, and that the N-terminal

amino acid sequence of phytases in wheats which do not depend on their cultivated variety are identical.

Acidic and alkaline phytases have been identified in plants. The first group is most active around pH 5.0 and includes those from seeds of soybean (Gibson & Ullah, 1988), maize (Laboure *et al.*, 1993) and pollens of *Lilium longiflorum* (the pH 5.0 phytase) (Lin *et al.*, 1987) and *Petunia hybrida* (Jackson & Linskens, 1982). The second group is most active around pH 8.0 and includes those from seeds of *Phaseolus vulgaris* (Scott & Loewus, 1986), *Pisum sativum* (Scott & Loewus, 1986) and pollens of *Typha lattifolia* (Hara *et al.*, 1985) and *L. longiflorum* (Scott & Loewus, 1986; Lin *et al.*, 1987). The phytases from wheat bran, PHY1 and PHY2, belong to the first group, since their optimum pHs were 5.5 and 6.0, respectively. In general, acidic phytases from plants show a low-specificity against various phosphorylated compounds and are regarded as a kind of acid phosphatase rather than phytase. PDW1 and PDW2, which were isolated in the previous study (Nakano *et al.*, 1997), exhibited high activity against various compounds in a similar manner as other acidic phytases. However, PHY1 and PHY2 from Nourin#61, a domestic variety, had relatively high specificity; PHY1 especially showed the highest activity on IHP. The K_m values for IHP of various phytases have been reported to be from 2.64 μM to 570 μM (Nagai & Funahashi, 1962; Lim & Tate, 1973; Hayakawa *et al.*, 1989; Laboure *et al.*, 1993; Gibson & Ullah, 1988; Ullah & Gibson, 1987; Greiner *et al.*, 1993; Scott & Loewus, 1986), and the K_m values of PHY1 and PHY2 were much lower than others. This shows that PHY1 and PHY2 have high affinity and specificity toward IHP. We hypothesize that the conformation of each of the phytases is different each other, and that the active sites of PHY1 are more specific for IHP than those of other phytases from wheat bran. In addition to studying the specific activity of phytases from wheat bran, we need to make a conformational study of them.

Pi normally inhibits the phytase activity, and the K_i value for Pi of various phytases has been described from 28 μM to 11.9 mM (Ullah, 1988; Gibson & Ullah, 1988; Greiner *et al.*, 1993; Lim & Tate, 1973). The K_i values for Pi of PHY1 and PHY2 were higher than others except *E. coli* (11.9 mM). Therefore, PHY1 and PHY2 exhibit lower inhibition compared to those of other phytases except *E. coli*.

Nagai and Funahashi (1962) described that the phytase from wheat bran is inhibited by Hg^{2+}, Ag^{+}, Cu^{2+}, Fe^{3+}, Zn^{2+} and F^{-}. These ions also exerted an inhibitory effect on PHY1 and PHY2. Gibson and Ullah (1988) reported that phytase activity from the cotyledons of germinating soybean seeds is stimulated by Mg^{2+}, Ca^{2+}, Mn^{2+} and Fe^{2+}. But PHY1 and PHY2 were not activated by metal ions.

The optimum temperature of PHY1 and PHY2 was higher than their stable temperature. Molecular movement generally depends on temperature, high temperatures lead to high movement, but temperatures greater than 50 to 60°C rapidly inactivate most enzymes by denaturing the protein. Molecular movement of phytase might be activated from 45 to 50°C for up to 5 or 10 min, but the protein is denatured after 15 min, and The optimum temperature of various phytases has been reported to be around 55°C, and that of PHY2 was close to this. That of PHY1 was slightly lower, but higher than that of PDW1 and PDW2. Thermal stability of PHY1 and PHY2 was obviously lower than that of PDW1 and PDW2. The reason is not clear but may be related to the season in which wheat seeds are sown (Nourin #61 is sown in fall, but DNS and WRS are sown in spring).

Bioavailabilities of hydrolysis products of IHP were reported recently (Spiers *et al.*, 1996; Kim *et al.*, 1996; Tarnow *et al.*, 1996), and are expected to have positive biological and chemical effects for medical and food industries. A comparative study of phytases revealed that those from domestic Nourin #61 have high affinity for IHP and show low-sensitivity for inhibition by Pi. Therefore, we conclude that the phytases are very useful enzymes for hydrolyzing IHP, and for the production of IP1-5 *in vitro*. Microbial phytases have also recently been added to the diet of growing pigs or poultry to improve the availability of phosphorus. Feeding a diet with wheat bran phytases is thus believed to be beneficial for animals with simple stomachs, such as microbial phytases. Using the phytases is developing a new utilization of wheat bran which is usually disposed of as industrial waste.

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